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ORIGINAL PAPER

The characteristic change in the distribution of S-100 immunoreactive folliculostellate cells in rat anterior pituitary upon long-term estrogen treatment is prevented by concomitant progesterone treatment

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Abstract The presence of folliculostellate cells in the anterior pituitary was described 49 years ago. These cells give about 10% of the whole cell population and through their long processes they provide intrahypophyseal communication. The folliculostellate cells contain S-100 protein. Its immunostaining was used to identify these cells. It was previously found that the diethylstilbestrol treatment basically influences the morphology and function of the trophic hormone secreting as well as the folliculostellate cells. In the present experiment, we have studied whether a concomitant progesterone treatment can prevent or attenuate changes caused by diethylstilbestrol treatment in the distribution of folliculostellate, prolactin, and GH cells. Diethylstilbestrol alone induced the appearance of prolactinomas. Inside the prolactinomas, folliculostellate cells were scattered but outside the prolactinomas they formed a demarcation line. Inside the prolactinomas, there were only a few growth hormone immunoreactive cells but they surrounded the prolactinomas in a ring-like pattern. When diethylstilbestrol was implanted with progesterone, the changes being characteristic for diethylstilbestrol treatment, could not develop. Concomitant progesterone influence prevented morphological changes in the anterior pituitary. Progesterone alone had no effect. In accordance with the formation of prolactinomas, the plasma prolactin level was very high in diethylstilbestrol treated rats. Concomitant progesterone treatment prevented the effect of diethylstilbestrol. Progesterone alone did not influence the prolactin level. GH levels did not significantly differ in any groups.

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Introduction

The presence of non-hormone secreting cells in the anterior pituitary was first described about 40 years ago [1]. It was observed that these cells did not contain secretory granules, therefore they were named chromophobes. These cells contributed to the regeneration of the anterior pituitary as it was observed in the gland implanted into the hypophyseotrophic area of the hypothalamus. Later on it became evident that the chromophobes represented a heterogeneous cell population. They were classified as folliculo-stellate (FSCs), follicular, marginal, degranulated hormonal, mesenchymal, and immune cells [2].

FSCs and follicular cells (FCs) represent stellate forms having long processes [3] and they are connected to each other and to hormone producing cells by gap junctions which provide quick communication and concerted action between the cells [4]. Yamashita et al. [5] demonstrated that there is a well-defined difference between the two cell types. FSCs mainly contain S-100 protein and the FCs mainly contain cytokeratin. Immunostaining of these proteins is a suitable method to localize the two cell types. In some cases, co-localization between the two immunoreactivities was demonstrated. Glial fibrillar acidic protein (GFAP) is an important component of the fibrous astrocytes in the brain but a few GFAP-positive cells were also observed in the anterior pituitary. Marginal cells are present between the anterior and intermediate lobes and they also contain nestin [6].

In the last two decades, more attention has been paid to FSCs and FCs than before. In the literature, most of the

papers do not make difference between the two cell types. It seems that both cell types produce several factors that influence the cell proliferation and the hormone production of the trophic hormone secreting cells by paracrine manner [7, 8]. These factors are the following: interleukin 6 [9], vascular endothelial growth factor (VEGF) [10], follistatin [11], basic fibroblast growth factor (bFGF) [12], chromogranin A [13], deiodinase (D2) and thyroid hormone transporter 8 (MCT8) [14], clusterin (helps the ingestion of apoptotic endocrine cells through megalin containing receptors), megalin [15].

It is well known that trophic hormone production is regulated by hypothalamic releasing and inhibiting hormones through the portal circulation. Besides hypothalamic factors, hormones of peripheral endocrine organs, among them sexual steroids, also regulate the function of both hormone producing or non-hormone producing cells. Long-term estrogen treatment induces proliferation of prolactin (PRL) cells, prolactinaemia, and formation of prolactinomas [16, 17], while it extremely depresses the number of luteinizing hormone (LH), follicle stimulating hormone (FSH), and thyroid stimulating hormone (TSH) cells and does not influence the adrenocorticotrop hormone (ACTH) cells [18]. With the use of hemolytic plaque assay it was demonstrated that a chronic estrogen treatment increased the percentage of PRL secreting cells and the amount of secreted PRL; however, there was a decrease in both the percentage of growth hormone (GH) secreting cells and the amount of secreted GH. Following the hemolytic plaque assay, in situ hybridization revealed that estradiol β (E2) increased the prolactin mRNA while it decreased the GH mRNA [19]. This research group did not find any co-localization between GH and PRL expression in control male rats; however, in E2-treated animals, about 10% of PRL secreting cells contained GH mRNA as well. It was also demonstrated that the effect of estrogen is mediated through α-estrogen receptor [20].

Susceptibility to E2 varies among strains of rats. The Fischer rats are more sensible to E2 than Sprague–Dawley rats. In vitro in a co-culture of Fischer and Sprague–Dawley derived lactotropes, Fischer FSCs stimulated the mitogen action of E2; however, Sprague–Dawley FSCs failed to stimulate it [21]. In this in vitro model, the FSCs were exposed to estrogen for a short time.

Protective effect of progesterone (P) on estrogen-induced undesired changes was observed about 20 years ago. It was demonstrated that P was able to reduce the size of estrogen-induced mammary tumor [22]. The protective role of P is also used to prevent preterm birth when applied as vaginal suppository [23]. Genazzani et al. [24] analyzed how P and various synthetic progestins modulate the effect of estradiol benzoate (EB) on hypothalamic gonadotropin-releasing hormone (GnRH) and on pituitary LH and PRL concentrations in ovariectomized rats. It was found that P and norethisterone enanthate (NET, synthetic progestin) reversed the EB-induced hypothalamic GnRH depression and elevated its level. Both P and NET blocked the EB-induced increase of pituitary LH, but plasma LH levels remained high. Progestins alone did not influence the PRL levels but reversed the EB-induced increase.

In clinical practice, long-term sexual steroid treatment as contraceptive medicament was suggested about 70 years ago [25], and this method is usually used still today. Estrogens are also used as replacement therapy after surgical removal of ovaries or in menopause or for treatment of human males with prostatic carcinoma. In these patients, estrogen alone depressed the FSH release, and addition of P was able to induce FSH peak [26]. However, the effect of concomitant P administration on the morphological changes in the anterior pituitary caused by estrogen treatment in long-term in vivo experiments is not clarified.

The aim of our present experiment was to study the distribution of S-100 immunopositive FSCs in the anterior pituitary of Sprague–Dawley male rats after a long-term estrogen treatment (5 months), which is able to induce micro- and macro-prolactinomas. The relation of PRL and GH cells to S-100 immunopositive cells was also examined. Then we studied whether a concomitant P treatment could prevent estrogen-induced changes. The reason for choosing Sprague–Dawley rats for our experiments was that human patients are probably nearer to a rat strain with a low susceptibility to E2 than to that of extremely high susceptibility. For steroid treatment, silastic capsule was filled with diethylstilbestrol (DES), DES + P or P alone. Upon the effect of body fluids, the steroids can gradually penetrate the wall of the capsule. In this way, both DES and P implants can maintain a continuous long-term hormone level in the experimental animals. Till the end of the long experimental period, PRL and GH plasma levels were also measured from the trunk blood to verify the steroid influence.

Results

Distribution of PRL and GH cells in control and sham-operated rats

In control animals, the PRL cells were cup-shaped and evenly distributed; however, there was a densely packed layer of the cells under the connective tissue capsule which caused more intensive fluorescence here than in the other part of the anterior lobe (Fig. 1a). The GH cells showed characteristic distribution. They were missing from the antero-medial pole of the gonadotrophic zone (Fig. 1b). Implantation of an empty capsule (sham

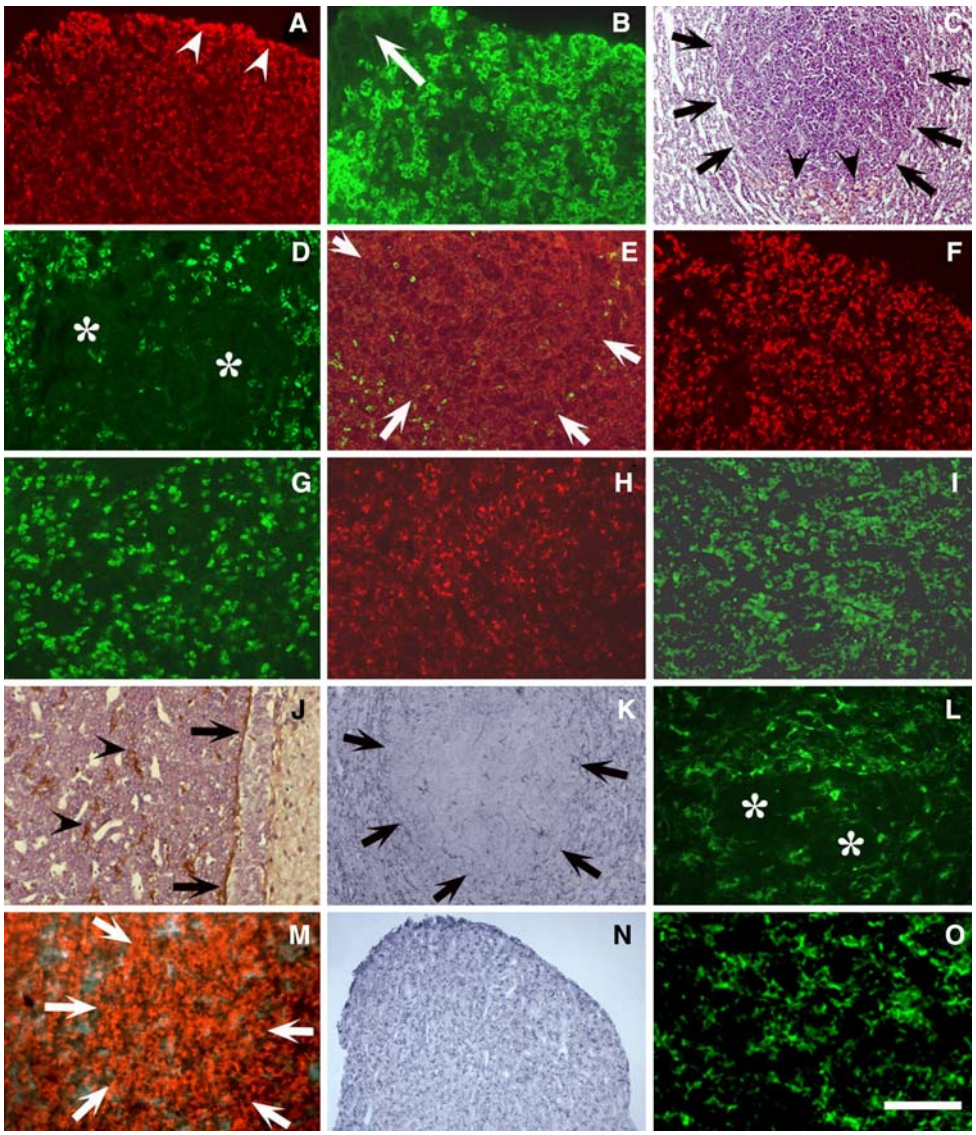

Fig. 1 Microphotographs show PRL, GH, and S-100 immunoreactivities in the anterior pituitary of intact **a**, **b**, and **j**, DES **c–e** and **k–m**, DES + P **f**, **g**, and **n**, and P **h**, **i**, and **o** treated male rats. **a** Red fluorescence indicates PRL immunoreactivity in the pituitary of a control rat. The cells show characteristic cup-shaped appearance. There is a densely packed layer of immunoreactive cells under the connective tissue capsule (white arrowheads). **b** Green fluorescence shows GH cells. In the anterior pituitary of control rats, GH immunoreactive cells are missing in the antero-medial pole of the gonadotrophic zone. White arrow shows the abovementioned region in the given section. In the other part of the anterior lobe, the GH cells are evenly distributed. **c** An adenoma in the anterior pituitary of a DES-treated rat. The slide is stained by hematoxylin-eosin. Black arrows outline the adenoma. Black arrowheads indicate a group of vessels in the neighborhood of the adenoma. **d** GH staining (green fluorescence) in a section of a DES-treated pituitary. In the adenomas (indicated by asterisks), GH cells are practically missing. **e** Double labeling for PRL and GH immunoreactivities in the pituitary of a DES-treated rat. GH cells are green, PRL cells are red. The adenoma is composed of PRL cells (prolactinoma), GH cells are located outside the prolactinoma, which is delineated by white arrows. **f** The distribution of PRL immunoreactive cells (indicated by red fluorescence) and **g** the distribution of GH immunoreactive cells (indicated by green fluorescence) in the anterior pituitary of a DES + P treated rat. **h** The distribution of PRL immunoreactive cells (indicated by red fluorescence) and (**i**) the distribution of GH immunoreactive cells (indicated by green fluorescence) in the anterior pituitary of a P-treated rat. **j** S-100 immunoreactive FSCs are evenly distributed in the anterior lobe of control rats. In the section, brown reaction product indicates the FSCs with long processes (black arrowheads). At the border of the anterior and intermediate lobes, FSCs form a boundary shown by black arrows. Background staining is hematoxylin-eosin. **k** Nickel intensified dark blue reaction product shows S-100 immunoreactive FSCs in the anterior pituitary of a DES-treated rat which surround an ovoid area resembling adenoma (delineated by black arrows).**l** In this slide, S-100 cells are stained by immunofluorescence method, the cells are green and surround small adenomas indicated by white asterisks. **m** Double labeling for S-100 (greenish-blue) and PRL (red) immunoreactivities reveals that S-100 cells are nearly missing from the prolactinoma. **n** S-100 immunostained FSCs (indicated by nickel intensified dark blue reaction product) in the anterior pituitary of DES + P treated rat and **o** FSCs (indicated by green fluorescence) in the anterior pituitary of a P-treated rat. In DES + P and P-treated rats, the distribution of PRL, GH, and FSCs was very similar to that of intact rats. Scale: 50 μm in **j**, **l**, **m**, and **o**; 100 μm in **a**, **b**, and **d**, **f–i**; 200 μm in **c**, **e**, **k**, and **n**

operation) did not alter the distribution of PRL and GH immunoreactive cells.

Distribution of PRL and GH cells in DES, DES + P and P-implanted rats

DES implantation induced cell proliferation and formation of adenomas. These adenomas had smaller–larger diameter. Figure 1c shows a large adenoma in a slide stained by hematoxylin-eosin. In the neighborhood of the adenomas, many small vessels were observed. The distribution of GH cells was strikingly different from the controls. They were missing from round-shaped areas (Fig. 1d). Double labeling for PRL and GH revealed that the round areas were prolactinomas and GH cells were nearly missing from these areas (Fig. 1e). When DES was implanted together with P, characteristic changes, which were caused by estrogen in the distribution of PRL and GH cells, were not observed while the shape of PRL cells was a little different from the controls. The immunoreactive material filled up the cells; they lost their cup-shaped pattern (Fig. 1f). GH cells did not show any striking changes in their shape. They were similar to controls (Fig. 1g). P alone did not influence either PRL (Fig. 1h) or GH staining (Fig. 1i).

Distribution of FSCs in control and sham-operated rats

In control and sham-operated animals, the S-100 immunoreactive cells (FSCs) were evenly distributed inside the anterior lobe; however, at the border of the anterior and intermediate lobes, they formed a dense layer (Fig. 1j). The S-100 cells were stellate with shorter–longer processes.

Distribution of FSCs in DES, DES + P and P-implanted rats

In the anterior pituitary of DES-treated rats, S-100 immunoreactivity was nearly missing in several round areas (Fig. 1k, l). In many cases, FSCs formed concentric layers surrounding these areas (Fig. 1k). Double labeling for S-100 and PRL immunoreactivities revealed that these areas contained PRL immunoreactive cells forming prolactinomas (Fig. 1m). The distribution and shape of FSCs in DES + P and only P-treated animals did not differ from control rats (Fig. 1n, o).

PRL and GH plasma levels in the animals of various experimental groups

Figure 2 shows PRL and GH levels in the trunk blood that was collected at the end of the experimental period. As it was expected, DES treatment extremely enhanced the plasma PRL level; it was more than 55-fold higher than in intact control rats. Concomitant P treatment prevented enhancement of PRL level. Empty capsule implantation and P alone had no significant effect (Fig. 2a). The various treatments did not significantly influence the GH levels (Fig. 2b).

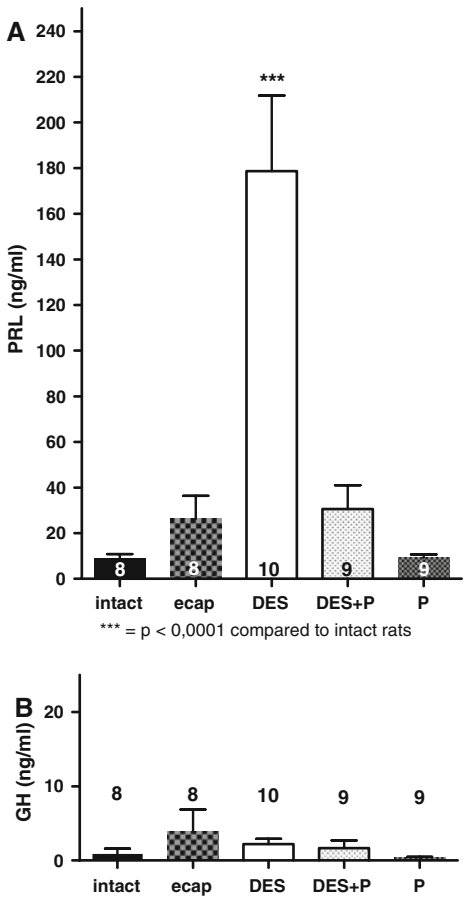

Fig. 2 The graphs show PRL and GH plasma levels in the trunk blood of animals of various experimental groups 5 months after implantation. The numbers in or above the columns indicate the number of animals included in the various experimental groups. DES extremely enhanced the PRL levels compared to intact and empty capsule implanted rats. Concomitant P treatment prevented this effect. P alone did not influence PRL levels. *Abbreviations*: ecap, empty capsule; DES, diethylstilbestrol; *P*, progesterone

Discussion

In the present experiment, it was found that a 5-month-long DES treatment induced hypertrophy of lactotropes, appearance of prolactinomas, and enhanced plasma prolactin level. There was a marked change in the distribution of S-100 immunopositive FSC cells and GH immunopositive somatotropes. Both cell types disappeared from the prolactinomas but the prolactinomas were surrounded by FSCs and GH cells from outside. In the other part of the

anterior pituitary, both cell types were evenly distributed. The periphery of the prolactinomas was highly vascularized. Concomitant P influence prevented morphological changes in the anterior pituitary and elevation of plasma prolactin levels. The distribution of FSCs and GH cells was very similar to those of in intact and empty capsule implanted rats. P treatment alone did not affect either examined parameters.

The question arises how estrogen induces and P prevents the abovementioned changes. They must be carried out through specific receptors. It is well established that the anterior pituitary is a target tissue for ovarian steroids. Besides the brain and other peripheral tissues, both estrogen and progesterone receptors are present in the anterior pituitary and both estrogen and progesterone can act locally on pituitary cells. Two types of estrogen and progesterone receptors were identified.

Estrogen receptor alpha (ERα) was cloned and characterized by Koike et al. [27], and estrogen receptor beta (ERβ) was cloned by Kuiper et al. [28]. In a recent publication it was found that ERα is localized in lactotropes of the largest number, and in somatotropes, thyrotropes, and gonadotropes of decreasing number. The number of ERβ was much lower and it was identified in somatrotropes, lactotropes, and gonadotropes [29]. Both receptor immunoreactivities were present in the nucleus, rough endoplasmic reticulum, secretory granules, and in the free cytosol. The intracellular localization depended on the stage of estrous cycle.

The mechanism how the estrogen stimulates the cell proliferation in the anterior pituitary was thoroughly examined [30, 31]. It was found that estrogen through ERα induces production of TGF-β3 from lactotropes and this stimulates the bFGF production of FSCs which has a proliferative effect on lactotropes. ERα and β knock-out experiment do not support the exclusive role of ERα. Mice with disrupted ERα display gonadal maldevelopment, consequent elevated gonadotropins, low sexual steroid levels, infertility and disturbed sexual behavior, and very low but detectable PRL levels [32]. This latter observation well correlates with the results which give evidence for lactotrop cell differentiation in ERα knock-out mice [20]. On the basis of these data, the hypothesis as to which ERβ may also be involved in this process may be accepted.

Progesterone receptors, both A and B isoforms, were demonstrated in the anterior lobe using RT-PCR technique [33] and immunohistochemistry [34]. The predominant form is the A isoform (PRA). In rat, PR immunostaining was seen in the nuclei of gonadotropes. The mechanism how the concomitant P treatment prevents the estrogen induced proliferation is not fully explored. Calderon et al. [35] used ovariectomized immature rats to analyze the action of P in response of anterior pituitary and hypothalamus to estradiol exposure. Estrogen induced the nuclear accumulation of estrogen receptors and the appearance of progesterone receptors reaching a peak at 12 h, and then declined to a plateau near the control level. If P was administered to the animals at the peak of progesterone receptors, subsequent nuclear accumulation of estrogen receptor caused by estradiol injection was suppressed. This was observed only in the anterior pituitary, not in the hypothalamus. They concluded that P affected the response to estradiol in the pituitary gland by a well-defined temporal pattern and using a same protocol it had no effect in the hypothalamus.

It seems that there is a cross-talk between estrogen and progesterone receptors. In our protocol, we ensured a consistently higher blood level of both estrogen and progesterone than in controls by setting in the silastic capsules for 5 months. It is not known how the estrogen and progesterone receptor levels changed during this long period. And there are no data in the literature describing such a long-term experiment.

There may be several explanations for the protective effect of P. (i) PRA is present in gonadotropes. P binding may stimulate these cells to influence the estrogen binding to lactotropes and to prevent the production of TGF-β3. (ii) Estrogen receptors in lactotropes may have progesterone responsive elements and progesterone can bind directly to these receptors to prevent the production of TGF-β3. (iii) The effect of P through the hypothalamus is not excluded. But the abovementioned experiment [35] indicates that P acts on the pituitary locally rather than through the hypothalamus. This conception is supported by the following observations. (i) In human adenoma cell culture, 17β-E2 had a stimulatory and P had an inhibitory effect on the cell growth [36]. This effect did not depend on the hormone content of the cells. (ii) P reduced the weight of DES-induced pituitary tumor, the level of nuclear estrogen receptors and cytosolic progestin receptors, and serum PRL level in rat [37].

It is also well established that estrogen stimulates angiogenesis in the adenomatous anterior pituitary. The rat anterior pituitary receives its blood supply via the hypophyseal portal circulation. It was demonstrated by Elias and Weiner [38] that estrogen-induced tumorigenesis of the anterior pituitary is associated with the development of a direct arterial blood supply. These vessels carry systemic blood in which dopamine is missing. The loss of dopaminergic inhibition in concert with estrogen stimulation may lead to tumor formation.

In dopamine D2 receptor knock-out mice, the pituitaries showed increased protein and mRNA of VEGF, suggesting that dopamine inhibits its pituitary expression. Double labeling revealed that VEGF is present in FSCs [39].

The phenomenon that the FSCs are missing from the prolactinoma cannot be explained by the data available in the literature. It is possible that the growth of prolactinoma (facilitated by FSCs) is not interstitial but appositional, and the FSCs play a main role which are concentrically arranged around the prolactinoma. Shimon et al. [40] described the presence of FGF-4 encoding gene (heparin-binding secretory transforming gene, hst) around the newly formed vessels outside, but not inside, the human prolactinomas. They also demonstrated the proliferative effect of hst gene transfection on cultured GH4 cell [41], indicating that FGF-4 (which is probably produced by FSCs) may be involved in the proliferation of lactotropes.

Materials and methods

Animals

Sprague–Dawley male rats were used for the experiment. The newborns were separated from the mothers at 21 day of life and then fed with standard lab chow (Gödöllő, Hungary) and water ad libitum. They were kept in a light (light on at 5 h and off at 19 h) and temperature (22 ± 2°C) controlled vivarium. The treatment of the animals was in accordance with the rules of the "European convention for the protection of vertebrate animals used for experimental and other scientific purposes", Strasbourg, 1986. Our protocol was approved by the Department of Animal Health Care, Budapest. Permission number: 37/1999. When the animals were 25-day-old, they received DES or DES and P or P containing silastic capsules implanted into the subcutaneous tissue of the back of the neck.

Preparation of the hormone containing capsules

Silastic capsules (id 1.55 mm, od 3.13 mm, length 10 mm) (Dow Corning Corporation, Midland, MI) were filled with DES (Sigma, St. Louis, MO) or P (Sigma) and the ends of the capsules were sealed by Szilorfix (Finomvegyszer Szövetkezet, Budapest, Hungary), a silicon-based glue. After 1 day of drying, the capsules were used for implantation.

Experimental groups

1. Intact rats
2. Sham-operated rats (they received empty capsule sealed with glue)
3. DES-implanted rats
4. DES + P implanted rats
5. P-implanted rats

Twenty male rats were included in the morphological experiment and 88 animals were used for measuring plasma PRL and GH levels. The survival time after implantation was 5 months.

Immunohistochemistry

At the end of the experiment, the animals were anesthetized by ketamine-hydrochloride (75 mg/100 g bw) (Sigma, St. Louis, MO) and perfused by 4% paraformaldehyde (Merck, Darmstadt, Germany) in potassium phosphate buffer (KPB) (pH 7.4, 0.1 mol). The components were purchased from Sigma (St. Louis, MO). Pituitaries were removed and postfixed overnight. After washing in KPB, the pituitaries were immersed in ascending sucrose solution (10–20–30%), then frozen on dry ice. Five parallel series of 20-μm-thick sections were cut on cryostate (Shandon, Pittsburg, PA). One series was stained by hematoxylin-eosin to demonstrate the presence of adenomas; four series were used for immunohistochemistry. The trophic hormones were visualized by indirect fluorescence technique. S-100 was stained using ABC Kit. The final complex was visualized by DAB (diaminobenzidine hydrochloride) or nickel-intensified DAB reaction; in some cases, S-100 was also stained by indirect fluorescence method. When just DAB was used for visualizing S-100 immunoreactivity, the background was stained by hematoxylin-eosin. The following primary antisera were used in our experiment: GH antiserum (dilution 1:500) was raised in guinea pig and obtained from the National Hormone and Peptide Program (NHPP), NIDDK, and Dr. Parlow. Prolactin antiserum (dilution 1:1000) was prepared in rabbit and characterized by DeMaria et al. [42]. S-100 (dilution 1:5000) was raised in rabbit and purchased from DAKO Corporation (Carpinteria, CA). Fluorescent labeled secondary antibodies were the following: rabbit anti-guinea pig IgG conjugated to FITC (DAKO A/S, Glostrup, Denmark), goat anti-rabbit IgG conjugated to FITC (DAKO A/S, Glostrup, Denmark). Prolactin fluorescent labeling was carried out by ABC technique and streptavidin Alexa 546 (Vector Laboratories, Burlingame, CA). Vectastain ABC Elite Kit was also purchased from Vector Laboratories (Burlingame, CA). DAB (diaminebenzidine hydrochloride) was purchased from Sigma. Nickel-ammonium sulphate was purchased from Reanal (Budapest, Hungary).

Radioimmunoassay (RIA)

The animals were decapitated in the morning at 10 h at the end of 5-month experimental period. The trunk blood was collected, its coagulation was prevented by EDTA (ethylene diamine tetraacetic acid Na_2 salt; Serva, Heidelberg,

Germany). Then the blood was centrifuged at 4°C. Plasma was stored at −20°C until determination of PRL and GH levels. Iodination was carried out by Chloramine T method. Separation of the free and bound antigens was made by double antibody method. Both hormone kits were obtained from the National Hormone and Peptide Program (NHPP), NIDDK, and Dr. Parlow. Hormone levels were expressed in ng/ml plasma. The mean of two parallel determinations for each animal was subjected to one-way analysis of variance (ANOVA) followed by a Student's *t* test.

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